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THE SIGNIFICANCE OF THE WHITE BLOOD CORPUSCLES IN ANTHRAX INFECTIONS

/Following is a translation of an article by F. de Moulin, of the Department of Preparation and Control of Antisera and Vaccines of the Veterinary Institute in Buitenzorg, in the Dutch-language publication Nederlandsch-Indische bladen voor Diergeneeskunde en Dierenteelt (Netherlands-Indies Journal of Veterinary Medicine and Animal Breeding) Vol 49, 1937, pp 199-208./

Notwithstanding the extensive knowledge obtained till now of anthrax one meets from time to time problems that are not sufficiently clarified or on which different authors do not agree. As anthrax vaccinations are so prominent in the East Indies it is very important to get a clear insight into the course of the subcutaneous infection with a virulent infectious matter and the subcutaneous application of vaccine. The part the leucocytes play in those reactive inflammations is by different investigators not always unanimously judged and the phagocytosis of live anthrax bacilli is thought possible by one and absolutely denied by another, or said to be possible only with dead infectious matter. The original opinion of Metchnikoff that virulent live anthrax bacilli can be subject to phagocytosis by leucocytes is doubted by many. Even that the virulent anthrax bacilli would have a certain chemotactic influence on the leucocytes is not quite unanimously accepted. Some accept this only in the organisms of anthrax-insensitive animals, or exclusively with weakened bacteria species; in this case anthrax vaccines in for the disease-susceptible individuals. With virulent infectious matter the leucocytes are more kept on a distance than attracted. Although maybe phagocytosis of virulent bacilli was not seen, it could not be denied that in the vaccination region strong leucocytosis and annihilation of many inoculated micro-organisms happened. This extra-cellular deterioration would not have been caused by leucocyte products, but by antibacterial properties of tissue liquids. Later, Bail, Preisz, Weil, Tsuda, among others, have shown that anthrax bacilli in vitro brought together with leucocyte-containing serum became victims of extra-cellular lysis caused by a strong bactericidal matter excreted by the white blood corpuscles.

It is not necessary as an introduction to quote the extensive literature on this subject. It is enough to know that who studies the anthrax immunization cannot find in the literature convincing proof of the influence of leucocytes on the virulent or weakened infectious matter in animal organism, immunized before or not. To clarify this, systematic research is done. It goes without saying that a histological investigation must be the basis of the research. The microscopic examination of the local tissue reactions after a subcutaneous inoculation of virulent or weakened microbes in anthrax-sensitive or previously immunized

experimental animals, should give an insight into the part played by the leucocytes.

To be sure to make histological slices exclusively of the place of vaccination, the edge or point of the ear of sheep and rabbits was taken. The to be injected bacteria suspension, rinsed off a 24 hours old agar culture with physiological salt solution, was sucked in a syringe; 0.25 to 0.50 cc was injected subcutaneously, and after times varying from 4 to 24 hours the vaccination places were, after anaesthetising, cut out for histological examination. After alcohol fixation of the tissue, paraffin slices were made and colored in the usual way, as well as by the Giemsa method.

The bacteria used were: the very virulent Poerwakart species, an apathogenic culture of this species made for rabbits, the B.R.S. and P.G.V. vaccines, usual in the Indies, and the Carbozoo vaccine known in Europe.

The following system of vaccination of experimental animals was practiced:

I. Killed virulent bacilli in normal rabbits.
II. Live bacilli of the above-mentioned species in normal rabbits.
III. The same in previously immunized rabbits and sheep.

I. Killed virulent bacilli.

To kill the virulent bacteria a concentrated suspension of a 24-hours-old bacilli culture on agar in physiological salt solution was kept one hour at boiling temperature. Already 4 hours after inoculation of this suspension a strong hyperaemia shows, followed by edema. The connective tissue and capillar endothelium are first subject to a proliferative stimulus, but show later in the course of the process a vacuolation and a fatty degeneration. The leucocytes concentration is strong, and with the lymphocytes they form a wall around the bacteria center. After 24 hours many of these cells are destroyed, showing lyses and nucleus crumbling. Only a lot of dead bacilli are to be seen, different from live ones, as later will be described, because after Giemsa-coloring they are not blue, but light-red. Amid the leucocytes are everywhere rests of bacilli but it is striking that even of the dead bacilli so very few are cleared away by means of phagocytosis.

II. Vaccination with live infectious matter.

In principle the microscopic picture of the infection with virulent live bacilli looks like the picture of the dead bacilli, but the tissue-reaction is much more intensive. Also the cell destruction has increased enormously. Unexpected is the appearance of eosinophile leucocytes. Already after four hours the inoculated and culture bacilli colored with Giemsa blue have acquired capsules which they keep in the further course of the process. Although leucocytes and lymphocytes concentrate in great quantity, phagocytosis is nowhere seen nor extra-cellular destruction,

and in only a few leucocytes there are spores.

The anthrax species made avirulent create only light inflammation symptoms, but the local hyperleucocytosis is strong. The good colorable bacilli form in rabbits no capsules and at the same time an enormous phagocytosis, as well as an extra-cellular destruction is to be seen. In forming morphological different degeneration products, with no similarity to the original shape, the bacilli are soon cleared away and after 24 hours nothing can be found anymore of the considerable quantity of inoculated microbes.

The vaccine species create a less strong inflammation than the virulent bacteria; the leucocyte emigration, however, is not less than in virulent infections but on the contrary even increased. Remarkable is the presence of eosinophile leucocytes which in greater quantity than in virulent infections participate in the process.

Only part of the vaccine bacilli acquire capsules, the other part is absolutely naked, also after 24 hours. The aggressive influence on the leucocytes is clearly visible in the destruction zone of these cells around the infection centre.

Phagocytosis is nowhere to be seen. On the contrary with the virulent species the vaccine bacilli are in considerable quantity extracellular destroyed as appears from the pictures of lysis, forming of shades and granular degeneration which are everywhere to be seen.

III. Live Infectious Matter in Immunized Animals.

As experimental animals rabbits and sheep were taken which after repeated inoculation with vaccine, and as to sheep also with virulent bacteria, were immunized against anthrax.

In rabbits the destruction of the inoculated fully virulent matter, a concentrated suspension, happens very quickly, and after nine hours practically nothing of the virulent Poerwakarta culture can be found. Phagocytosis is nowhere found with certainty so that the destruction must have happened exclusively extra-cellularly. The inflammation reaction is far less than in infection of non-immunized rabbits, and the leucocytes degeneration is less. The presence of eosinophile leucocytes around the infection centre is remarkable.

With the apathogenic culture made for rabbits; the inflammation is minute and there is no cell degeneration at all. The more is the destruction of the inoculated bacilli, which are cleared away by means of intra- as well as extracellular digestion. The leucocytes emigration comes more slowly, but is after some hours considerable; local eosinophilia is minute. After 24 hours a trace of the inoculated infectious matter can nowhere be found.

In immune rabbits nine hours after inoculation with vaccine no convincing bacilli rests are anymore to be found. The destruction happened so quickly that images of phagocytosis and even of extracellular digestion are lacking after nine hours. Symptoms of inflammation, hyperaemia, edema and leucocyte degeneration are still present so that the aggressive character of the bacilli is clear. The amount of eosinophile

leucocytes has, because of the immunization before, strongly increased. With the much swollen and vacuolated connective tissue- and endothelium cells they give quite the tissue picture that is described of subcutaneous injections of anaphylaxis-creating material.

The same experiments are repeated with immune sheep. The histological picture is quite the same as seen in rabbits. Growth and degeneration of connective tissue- and endothelium cells, emigration of leucocytes, and specially the presence of eosinophile cells, hyperaemia and light edema complete the character of inflammation.

Already four hours after inoculation of the virulent Poerwakarta culture the fine bacilli show different symptoms of degeneration. In case they are at the outside of the bacilli centre and in contact with the bactericidal matter in the tissue they show involution forms, swelling, winding, clavated thickening and coccus shapes of different thickness. In the same time the bacilli are less colorable. They may have capsules, but they exist a short time and disappear after granular degeneration and an irregular and considerable swelling. Already after four hours are with the capsules many bacilli on their way to destruction. After 24 hours no capsules exist anymore and the clearing away of the bacteria is far advanced. Not everywhere is this in the same phase of development and next to short segments without capsules and fine bacilli, there is everywhere amid the leucocytes a granular and filamentous detritus. Phagocytosis does not exist. Leucocyte degeneration is minute even in the bacteria centre. The great amount of lymphocytes seem to prove their participation in the process.

The vaccine bacilli inoculated in immune sheep create a local leucocytosis, far stronger than with the virulent species. Also there are many more eosinophile cells.

With these bacilli the forming of capsules is prevented and only in a few cases can the beginning of a capsule be found. These capsules disappear in a few hours because of granular degeneration, so practically it can be stated that immunity prevents capsule forming. Unlike in the virulent species, phagocytosis takes place everywhere, and the eosinophile leucocytes are also participating. In addition to this intracellular digestion a considerable extracellular digestion takes place and all stages of degeneration of the bacilli outside the leucocytes are found.

With the weak vaccine species (P.G.V.) the degree of phagocytosis is stronger than with the stronger (B.R.S.). The Carbozoo from Europe is in accord with the P.G.V. vaccine.

After 24 hours no one of the vaccine species shows bacilli rests in the place of inoculation.

From the above-mentioned facts the following practical conclusions can be made:

The tissue- and cell deterioration stands in relation to the virulence degree of the inoculated anthrax species. The leucocytes emigration of the vaccines is stronger than with virulent matter, and with the weaker P.G.V. and Carbozoo more so than with the stronger B.R.S. vaccine. This could be explained by the possible presence of aggresines in the more virulent species. Eosinophile leucocytes emigrate more in

vaccine inoculation than in virulent infections, preceding sensitising by means of vaccination increases the amount of eosinophile cells considerably. Here is something encountered also being established in anaphylaxis-creating material. Preceding immunization increases the phagocytosis of vaccine bacilli considerably and remains unchanged as to the virulent bacteria; the latter are neither in the normal nor in the immune animal subject to phagocytosis.

Vaccination increases the leucocyte emigration after a next inoculation of vaccine bacilli; a next inoculation with Poerwakarta anthrax does not act this way.

The virulent bacteria are in immune rabbits and sheep exclusively extra-cellularly destroyed, the apathogenic and vaccine bacteria are destroyed by both intra- and extra-cellular digestion.

Vaccination furthers both forms of leucocyte activity. The intensity of phagocytosis stands in relation to the diminished degree of virulence.

The forming of capsules seems to be in connection with the degree of virulence and is strongly checked in case of immunity, and the time during which the capsules manage to exist is only shortened by a few hours in case of immunization. Capsule bacteria in these experiments have never been subjected to phagocytosis, nor have the mitigated capsuled vaccine bacilli.

Another important problem is to investigate whether the leucocytes, although they do not destroy live virulent bacilli by means of phagocytosis, still excrete products causing extra-cellular digestion and how such products act on weakened bacteria.

Therefore it was tried by producing sterile abscesses in big domestic animals, normal as well as highly immunized, to obtain a leucocyte extract and to test the influence of this extract on anthrax cultures.

A normal cow and normal horse, a highly immunized horse and buffalo were injected under the skin of the neck with 5 cc of oleum terebinthinae depuratum. After 5 to 7 days abscesses had formed at the places of injection. The contents of the abscesses were gathered under precaution of sterility in little bottles and mixed with physiological salt solution kept a week under refrigeration. From time to time the mixture was strongly shaken. At last part of the mixture was put in sterile centrifuge tubes and rotated. Part of the clear liquid was heated in a double boiler to 60, 70 and 80 degrees centigrade. Two cc of the different leucocyte extracts from normal as well from immunized animals were added to 24 hours old anthrax cultures on agar and after 4.8 and 24 hours the results were examined on slides. The series of agar cultures were prepared from the apathogenic Poerwakarta species for rabbits and from the already mentioned vaccine bacteria.

The normal unheated leucocyte extract shows after four hours an unfavorable influence on the virulent bacilli, which show a swelling of the originally slender bacilli. After 24 hours the bacilli are to a certain extent polymorphous, oval; much swollen segments are next to slender bacilli often in an unreal capsule. Many of those bacilli with

changed shapes are much less colorable.

The influence of the normal leucocyte extract on the vaccine bacteria and the apathogenic culture is much clearer. Here less of a swelling is shown than a decline in dimension, so a deliquescence. All different shapes are to be seen and no similarity exists anymore with the original bacteria: There are coccus shapes and bodies resembling coli bacilli, shades, fine sticks and granula. The amount of bacteria which remained seemingly normal is far less than in the virulent species.

The unheated normal leucocyte extracts of cow and horse act in the above-mentioned same way.

In heating to 60 degrees all influence of the extract on the cultures is lost.

The unheated leucocyte extract of immunized animals shows a quite different influence. Added to the Poerwakarta bacteria after four hours there is a clearly visible digestion of bacteria. The bacilli still have their long chain connections, but they are less colorable. The bacilli bodies are granulated. Within 24 hours the granular deterioration continues till the long bacilli-filaments fall apart in little granula. Also a total loss of colorability, with or without swelling and vacuolation, may produce long lines of shades indicating the place of the previously unimpaired filaments. After 24 hours no bacillus is to be seen and the original culture, because of the leucocyte products, has changed into an undefined mass of detritus in which vague outlines of former filaments are recognizable. An intensive bacteriolysis cleared away the whole mass of bacteria.

The vaccine bacteria suffer the same influence as the virulent species, but the course of the lysis is much faster. After eight hours the original culture is already in a far advanced state of deterioration. Losing their homogene morphology the vaccine bacilli acquire all different shapes and the original shape becomes unrecognizable; cocci- and coliforms with rounded ends change into granular- and line form rests and are dissolved in 24 hours. In comparison with the bacilli of the Indian vaccines the annihilation of the Carbozoo is a little slower and shows a little more similarity to the involution forms of the virulent bacteria, granular and shadowy bacilli keeping their original sizes. For the vaccine bacteria the deliquescence seems to be more characteristic. It is peculiar that the Poerwakarta species made apathogenic shows more of the destruction forms of the original virulent bacteria and less of the deliquescence of the vaccine bacilli and therefore does not go through all the differences of form varieties during lysis.

By comparison, agar cultures were made from the detritus masses after 24 hours action of the normal and immune leucocytes. From the normal extract the growth was considerable after 24 hours, from the immune extracts far less and only scattered colonies appeared, doubtless originating from undamaged spores. This shows the difference in activity between the two leucocyte extracts.

Heating to 60 degrees removes the best part of the activity of the immune extract and heating to 80 degrees makes it totally inactive.

In the histological examination the activity of the lymphocytes had already been obvious, although much less than of the leucocytes. Consequently the question was asked how far they play a part in the annihilation of the bacteria. By chance a highly immunized buffalo had died of an intestinal disease, the lymph glands could be extirpated and under precaution of sterility ground fine in physiological salt solution and kept under refrigeration for one week. For comparison the same glands of a normal animal were treated in the same way. From time to time the mixtures were well shaken.

In the same way as has been described in the case of the leucocyte extracts heated and unheated lymphocyte extracts were added to agar cultures with the following results:

The reaction of the bacilli to normal and immune lymphocyte extract is clearly different. Normal lymphocytes have practically no influence on the virulent culture and only after 24 hours some loss of homogene coloring is seen in the filaments. Immune lymphocytes cause shadows, gaps in the filaments, and in many places fine granula are all that is left of the original bodies. Although considerably less than the leucocyte extract the lymphocytes product also possesses a bacteriolytic effect.

The normal lymphocytes show so much lytic capacity against vaccine bacteria that there is only little difference with the products of the immune lymphocytes. Heating to 60 degrees make both extracts lose their capacity. The bacteriolytical activity, fortified by immunization, explains the presence of the lymphocytes in the anthrax process. Although these cells have no ability for phagocytosis, they participate in the extracellular annihilation of infectious matter.

Summary

About the part played by leucocytes in anthrax infections contradictory experiences are often published, so that to get a clear insight in practical anthrax problems it is necessary to investigate this matter yourself.

In rabbits and sheep a series of vaccination processes are produced and a histological examination done. Dead and live virulent anthrax bacilli, an apathogenic culture made for rabbits, the vaccine species used in the Indies, and Carbozoo species known in Europe, are inoculated in the skin of the ear of normal rabbits and of immunized rabbits and sheep. The places of inoculation are cut out in 4 to 24 hours for histo-bacterioscopic examination. The results were as follows:

The leucocyte and lymphocyte emigration is in vaccine inoculation stronger than in virulent infection.

Killed virulent bacilli are seldom victim of phagocytosis, live virulent as well as vaccine bacilli are not subject to phagocytosis during the first 24 hours. There is only phagocytosis of the apathogenic culture.

Only part of the vaccine bacilli acquire capsules in normal rabbits and many bacilli do not get capsules, same as with the

apathogenic bacteria.

Capsule bacteria are never subject to phagocytosis. The observations of Sobernheim and others that virulent capsule bacteria are subject to phagocytosis could not be confirmed. This supports the opinion of Tsuda, Weil and others who also deny the possibility.

Previous immunization considerably aids the local leucocytosis after inoculation with virulent or vaccine bacteria, but the reaction of the vaccine is stronger.

Phagocytosis is not promoted by immunization and they are exclusively cleared away by means of extra-cellular annihilation. The vaccine bacilli are after immunization cleared away as well by means of phagocytosis as by extra-cellular digestion. This process is very rapid; already after nine hours the bacilli inoculated in immune rabbits have disappeared. This process takes 24 hours in immune sheep, the vaccine bacteria are here sooner annihilated than the virulent matter.

- END -